BINDING OF NONSUPPRESSIBLE INSULIN-LIKE ACTIVITY (NSILA) TO ISOLATED FAT CELLS: EVIDENCE FOR TWO SEPARATE MEMBRANE ACCEPTOR SITES

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1. Introduction

The biological effects of insulin and NSILA, a polypeptide purified from human serum, on adipose tissue and isolated fat cells have been shown to be identical [1–5]. Among these, carrier-mediated stimulation of glucose uptake across the fat cell membrane [2] and inhibition of epinephrine-induced cyclic AMP-release from adipose tissue [6] suggest that the mechanism by which both hormones act at the cell membrane level is also the same. Therefore, we expected that the primary step of action of insulin and NSILA on adipose tissue involves binding to the same membrane acceptor site. To test this hypothesis binding studies with ^{125}I-labelled insulin and NSILA were performed with isolated rat fat cells. The results are compatible with the presence of two membrane binding sites for NSILA on adipocytes, one which appears to be identical with the insulin acceptor, but with a lower affinity for NSILA, and a second one which displays specificity and high affinity for NSILA, but not for insulin.

2. Materials and methods

2.1. *Animals*

Male, normal fed Zbz-Cara (formerly Osborne-Mendel) rats, weighing between 100 and 120 g were used for all experiments. Fat cells were prepared from epididymal fat pads by the method of Rodbell [7] using crude collagenase (Worthington Biochemical Corp.).

2.3. *Experimental*

All NSILA preparations (specific biological activity ranging from 3.8 to 200 mU/ml, standardized in the fat pad assay using insulin as reference), reduced aminoethylated (RAE)--NSILA and whale insulin were kindly provided by Drs E Rinderknecht and R. E. Humbel. Glucagon was a gift from Elli Lilly, $ACTH_{1-24}$ (Synacthen) from Ciba Geigy, Basel; human growth hormone (HGH) was donated by Dr M. Zachmann.

NSILA (~ 200 mU/ml, 50–60% pure; [8]) and whale insulin (same amino acid sequence as porcine insulin) were iodinated according to the method of Hunter and Greenwood [9], modified as described earlier [10].

Incubation of the cells was carried out in 20 ml plastic tubes in a shaking water bath for 60 min at 24°C in Krebs-Ringer-bicarbonate buffer containing 1% human serum albumin (HSA; from the Swiss Red Cross, Bern) and 20 mg/100 ml of glucose. 1 ml of the incubation mixture usually contained between 20 and 30 mg of fat cells and between 0.5 and 1.0 μU (200 000–400 000 cpm) of ^{125}I-labelled NSILA or between 10 and 20 μU (200 000–400 000 cpm) of ^{125}I-labelled insulin in addition to the unlabelled peptides. At the end of the incubation the cells were filtered on Millipore filters (EAWP) under a soft vacuum and rinsed three-times with 3 ml of ice-cold Dulbecco buffer containing 0.1% HSA. The dried filters were counted in 5 ml of Instagel (Packard) in a liquid scintillation counter (Nuclear, Chicago). All samples were run in triplicate.

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3. Results and discussion

Fig.1 A shows the displacement of ^{125}I-labelled insulin by increasing concentrations of unlabelled insulin and by three NSILA-preparations of different purity. Specific binding to 20 mg of fat cells of ^{125}I-labelled insulin (= difference between binding in the absence and presence of 100 mU/ml of cold insulin) was 1.15 ± 0.33% (mean ± S.D. of 14 experiments) of the added radioactivity. Between 40 and 60% of the total binding accounted for non-specific binding (in the presence of 100 mU/ml of cold insulin). Half-maximal displacement was achieved by ~ 2 X 10^{-9} M insulin which is in good agreement with the data reported by Gammeltoft and Gliemann [11] and by Livingston et al. [12]. As observed earlier [13] NSILA displaced labelled insulin. The displacing potency of the three NSILA-preparations correlated reasonably well with their specific biological activities measured in the fat pad assay. 2 mU/ml caused near maximal displacement (> 90%) compared to the displacement observed in the presence of 100 mU/ml of cold insulin. However, the displacing potency of NSILA was only ~ 1/35 (half-maximal displacement at ~ 7 X 10^{-8} M NSILA) of that of cold insulin. A potency ratio of ~ 1/60 between NSILA and insulin was found when the stimulation of [1-^{14}C]glucose oxidation by the two peptides in isolated fat cells was compared (fig.1B: half-maximal stimulation by insulin: ~ 6 X 10^{-11} M; by NSILA: ~ 3.4 X 10^{-9} M).

As shown in fig.2, ^{125}I-labelled NSILA was bound

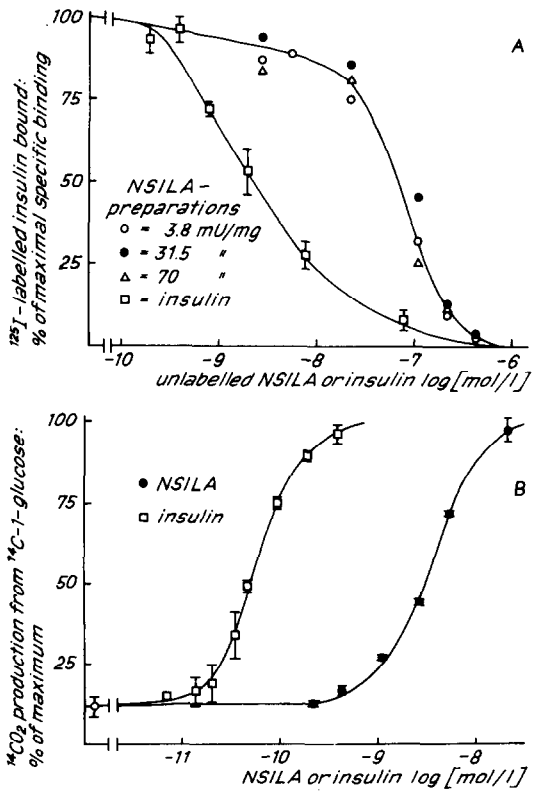

Fig.1. (A) Competition between ^{125}I-labelled whale insulin and increasing concentrations of unlabelled whale insulin or of unlabelled NSILA of different purity for binding to isolated rat fat cells. Incubations were carried out as described in materials and methods. Nonspecific binding in the presence of 100 mU/ml of cold insulin or of 2 mU/ml of cold NSILA has been subtracted from all values and the latter have then been expressed as percent of maximal specific binding. Each point represents the mean of at least 3 different experiments carried out in triplicates each. Bars give the SEMs, which have been left out for clearness on the NSILA curve. Molar concentrations of NSILA have been calculated on the basis of a molecular weight of 5800 and a specific biological activity for pure NSILA of 400 mU/mg [15]. (B) Stimulation of [1-^{14}C]glucose oxidation by whale insulin and NSILA (specific biological activity: 70 mU/mg) in isolated rat fat cells. Experimental procedure as described in Materials and methods. All values are expressed as % of maximal stimulation obtained at 7 X 10^{-10} M insulin. Each point represents the mean of 4 incubations; bars give the SEM. Calculation of molar concentrations of NSILA as in fig.1A.

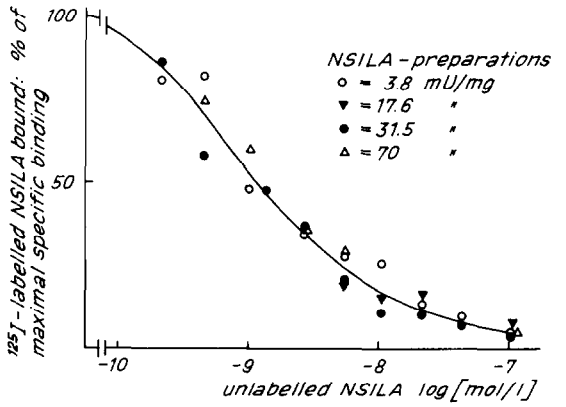

Fig.2. Competition between ^{125}I-labelled NSILA and unlabelled NSILA of different purity for binding to isolated rat fat cells. Incubation conditions are described in materials and methods. Each point represents the mean of 6–9 incubations. Nonspecific binding in the presence of 1 mU/ml of cold NSILA has been subtracted from all values. Calculation of molar concentrations as in fig.1A.

to isolated fat cells and displaced by cold NSILA. Binding of ^{125}I-NSILA was linear between 10 and 80 mg/ml of fat cells, equilibration of binding was reached after 20 min of incubation at 24°C (not shown). When ^{125}I-NSILA containing medium was used for a second binding experiment with fresh fat cells after a 60 min incubation in the presence of fat cells, no loss of binding was detectable (not shown). Hence, degradation of labelled NSILA by fat cells seems insignificant during the time chosen for the binding experiments (60 min).

Nonspecific binding determined in the presence of 1 mU/ml of cold NSILA was between 20 and 40% of the total radioactivity added. 100 μU/ml of cold NSILA caused more than 90% displacement of the specifically bound label. Specific binding of ^{125}I-NSILA was 0.46 ± 0.08% per 20 mg of fat cells (mean ± S.D. of 9 experiments). The displacing potency of four different NSILA-preparations agreed more or less with their specific biological activities determined in the fat pad assay. Half-maximal displacement was observed at approximately 10^{-9} M of cold NSILA, a figure which lies within the same range as the K_D-value determined in chick embryo fibroblasts [10]. Human growth hormone (HGH), ACTH and glucagon did not compete for binding of ^{125}I-NSILA, and reduced aminoethylated NSILA (RAE-NSILA), which is inactive in the fat pad assay, interfered with binding of ^{125}I-NSILA only at much higher concentrations (table 1). In contrast, insulin did not displace ^{125}I-NSILA, but rather consistently enhanced binding of the tracer (table 1). This finding has also been observed in the perfused rat heart [14], but remains unexplained at the present time.

The results obtained from the experiments shown in fig.1 and fig.2 suggest the presence of two different binding sites for NSILA in rat adipocytes: one which is likely to be identical to the insulin binding site, but which displays a much lower affinity to NSILA than to insulin, and another one which is specific for NSILA. Further evidence for the presence of two different binding sites was obtained by the experiments shown in fig.3: Fat cells were preincubated with or without an excess of cold NSILA (500 μU/ml). They were then washed and their remaining binding capacity for labelled insulin (fig.3A) and labelled NSILA (fig.3B) was determined. Prior to washing binding of ^{125}I-insulin in the presence of an excess of cold NSILA was

Table 1

Unlabelled peptides added	μg/ml of hormone protein added	Total binding of ^{125}I-NSILA cpm/ml (n = 3)	% of total binding
none	–	2220 ± 37	100
NSILA (3.8 mU/mg)	0.630[b]	500 ± 69	23
RAE-NSILA[a]	0.006[b]	2368 ± 184	107
RAE-NSILA[a]	0.063[b]	1790 ± 96	81
RAE-NSILA[a]	0.630[b]	1596 ± 67	72
human growth hormone	30	2053 ± 46	92
$ACTH_{1-24}$ (Synacthen)	25	2436 ± 147	109
Glucagon	0.400	2200 ± 94	100
Insulin	0.400	3326 ± 119	150

Fat cells (25 mg/ml) were incubated for 60 min at 24°C in the presence of ^{125}I-labelled NSILA (~ 0.5 μU/ml, ~ 300 000 cpm) together with the peptides given above. Binding of the tracer was measured as described in materials and methods.

[a] RAE-NSILA: reduced aminoethylated NSILA (specific biological activity in the fat pad assay before reduction and aminoethylation: 17 mU/mg.

[b] For NSILA the amount of hormone protein has been expressed in terms of pure peptide (specific biological activity ~ 400 mU/mg.

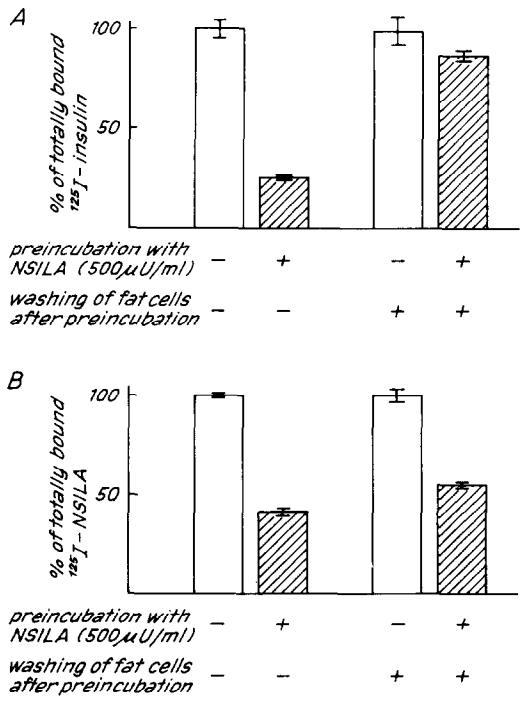

Fig.3. (A) Binding of ^{125}I-labelled insulin by fat cells preincubated with cold NSILA (specific biological activity 3.8 mU/mg) without or with prior washing after preincubation. Fat cells (22 mg/ml) were preincubated for 30 min at 24°C in Krebs-Ringer bicarbonate buffer/1% HSA. 1 ml of the same buffer containing ~ 10 μU of ^{125}I-labelled insulin was added and incubation was continued for another 60 min at 24°C. Controls were preincubated without or with 500 μU/ml of cold NSILA for 30 min; however, ^{125}I-insulin was added without prior washing of the cells. Bars give the SEM of 4 incubations. (B) Binding of ^{125}I-labelled NSILA by fat cells preincubated with cold NSILA (specific biological activity 3.8 mU/mg) without or with prior washing after preincubation. The experimental procedure was the same as in fig.2A, except that ^{125}I-labelled NSILA was added instead of ^{125}I-labelled insulin.

only 25% compared to the binding in the absence of cold NSILA. After washing the cells, ^{125}I-insulin was bound nearly to the same extent as in the absence of cold NSILA during preincubation.
This was different, when ^{125}I-NSILA was used in the second incubation: ^{125}I-NSILA binding remained depressed in spite of the washing procedure. These data are compatible with the following interpretation: NSILA appears to have a relatively low affinity for the insulin binding site from which it may be removed by washing. There seems to be a second binding site which binds NSILA much more tightly and from which it cannot be readily removed by the washing procedure. Some indirect evidence that rat fat cells contain a NSILA-binding site different from that of insulin has recently been obtained by Solomon et al. [16] and by Renner and Hepp [17]. Our competitive binding studies substantiate the findings of these authors.

It appears reasonable to assume that NSILA exerts its insulin-like effects on fat cells via the insulin receptor (1) because the biological potency ratio between NSILA and insulin (~ 1 : 60) lies within the same order of magnitude as the potency ratio for the displacement of ^{125}I-insulin (~ 1 : 35) and (2) because the ratios between the biological and the ^{125}I-insulin-displacing potency are also similar (insulin ~ 33 : 1, NSILA ~ 20 : 1). These findings are in contrast to those obtained with the rat heart where the comparison between receptor binding data and the biological potencies of insulin and NSILA suggests that NSILA acts via the NSILA binding-site [14]. It remains to be elucidated whether the NSILA binding site of adipocytes is 'non-functional' or if some as yet unknown effects are mediated through its interaction with NSILA.

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